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THE TISSUE DISTRIBUTION
OF EXOERYTHROCYTIC SCHIZONTS IN
SPOROZOITE-INDUCED INFECTIONS
WITH PLASMODIUM
CATHEMERIUM

A PART OF A DISSERTATION SUBMITTED TO
THE FACULTY OF THE DIVISION OF THE
BIOLOGICAL SCIENCES IN CANDIDACY FOR
THE DEGREE OF DOCTOR OF PHILOSOPHY

DEPARTMENT OF BACTERIOLOGY AND PARASITOLOGY

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THE TISSUE DISTRIBUTION OF EXOERYTHROCYTIC SCHIZONTS IN SPOROZOITE-INDUCED INFECTIONS WITH PLASMODIUM CATHEMERIUM

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In the development of malarial parasites in cells other than erythrocytes, designated "exoerythrocytic schizogony" by James and Tate,¹⁶ it was pointed out by Porter and Huff²⁰ that there seemed to be at least two distinct phenomena involved. *Plasmodium elongatum* exhibits morphologically similar schizonts in all food and blood-forming cells. Various other avian malarias show, in certain tissue cells and perhaps extracellularly, large schizonts morphologically distinct from the classical stages in erythrocytes. Huff and Bloom¹⁴ made a critical study of the types of cells infected by *P. elongatum*. The general neglect of such study on other species of *Plasmodium* has resulted in serious confusion in the literature as to the particular cells involved. With the purpose of clarifying this situation, if possible, an attempt has been made in the present study to determine, in a manner similar to that of Huff and Bloom,¹⁴ the tissue distribution of exoerythrocytic schizonts in infections with *Plasmodium cathemerium*. Huff and Bloom¹⁴ failed to find changes in the cellular localization of *P. elongatum* with the progress of infection. It was necessary, however, to investigate this possibility in the present study, especially since Kikuth and Mudrow¹⁷ had noted that in birds inoculated with

sporozoites of *P. cathemerium* exoerythrocytic schizonts appeared in the liver before they could be found in the brain.

Considerable study has been devoted to the occurrence of exoerythrocytic schizogony in various strains and species of malaria (See Porter and Huff²⁰). The findings, however, have been irregular. Since it appears that continued investigation of this question will be necessary to an explanation of the occurrence of the exoerythrocytic stages, they have been sought in a number of strains available in this laboratory. The present investigation deals with the occurrence of exoerythrocytic stages in different laboratory strains (section 1) and the changes in their distribution during the course of sporozoite-induced infections with *P. cathemerium* in the canary (section 2).

MATERIALS AND METHODS

Dry smears of peripheral blood and of the tissues of infected birds were stained with MacNeal's tetrachrome after methyl alcohol fixation. The routine examination of various strains of avian malaria for exoerythrocytic schizogony was made on dry smears of the liver and brain and occasionally of other tissues. The distribution of exoerythrocytic stages was studied in celloidin sections of tissues fixed in Zenker-formol and stained with Maximow's hematoxylin-eosin-azure II as used by Huff and Bloom.¹⁴ Early in the work wet-fixed tissue smears were used, but it was soon apparent that sections were necessary for most of the study. The method has important advantages. First, such fixa-

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tion and staining is essential to the identification of many of the cell types in which exoerythrocytic schizonts have been reported. Second, the fact that many parasites occur in ordinary endothelium can be conclusively demonstrated only in sections. Third, except in the brain, only sections show the numerous parasites in capillaries throughout the body.

Mosquito transmission was performed with various strains of *Culex pipiens* infected 10 or more days previously by feeding on birds whose peripheral blood contained abundant gametocytes. Early experiments indicated the danger, probably from embolism, of intravenous inoculation with ground whole mosquito thoraces, and the irregular infectivity of suspensions in normal salt solution. Therefore, the salivary glands were removed and placed immediately in heparinized whole normal canary blood. The mixture was ground in a small roller mill until dry smears of the suspension showed uniform distribution of the sporozoites. Each canary was inoculated intravenously with a quantity of this suspension representing 5 to 50 mosquitoes.

Most of the strains of malaria used are sufficiently identified in the text, but a few require description. The strain designations used here are the ones newly allocated by the Committee on terminology of strains of avian malaria of the American Society of Parasitologists. Strains which are no longer being maintained as laboratory strains were not allocated by the Committee and are referred to here as undesignated. In the designations the beginning numeral refers to the species (1 to *relictum*, 3 to *cathemerium*, etc.) and the letter to the strain originally isolated (*H* to Hartman, *M* to magpie, etc.). A description of this terminology is given by the Committee in the *Journal of Parasitology*

(June, 1942). Strains 3*M*1 and 3*M*2 were obtained from the magpie (3*M*) strain of *P. cathemerium* by intravenous inoculation of small numbers of parasites. They are somewhat less virulent than the parent strain. Two undesignated strains of *P. cathemerium* were found by Huff in English sparrows from Indianapolis, Indiana. Strains 3*H*1, 3*H*1-1 and 3*H*1-2 have been discussed by Huff¹³ under the designations, *N*, *U*, and *P* respectively. They were derived from the Hartman (3*H*) strain of *P. cathemerium*, strain 3*H*1 by single cell inoculation, strains 3*H*1-1 and 3*H*1-2 by mosquito passage of strain 3*H*1. Two other undesignated strains, apparently *P. relictum*, were found in laboratory canaries. Strain 3*T* of *P. cathemerium* is the woodthrush strain of Hegner and Wolfson,¹¹ designated *W* in their publication.*

Some explanation is necessary for the statistics used in the present study. The measure of reliability adopted is the probable error as a percentage of the number of parasites per square millimeter of tissue section. This is the type of measure used by Hartman,¹⁰ and by numerous investigators since then, for parasite counts in peripheral blood smears. However, a different approach is required here, since the sample unit is an area of tissue rather than a given number of cells. The probable error of a number of objects in a given area, as used in estimating the reliability of hemocytometer counts, is

$$P.E. = .6745\sqrt{n}$$

where "*n*" is the actual number of objects counted. If the probable error is to be "*c*" per cent of the number, we may substitute

* I am greatly indebted to Dr. Fruma Wolfson, of the Johns Hopkins University, for providing this strain.

$$\text{P.E.} = \frac{cn}{100}$$

giving

$$c = \frac{67.45}{\sqrt{n}}$$

Thus, the probable error is 10% of the number if 45 parasites are counted, 15% if 22 parasites are counted and 20% if 11 parasites are counted. The applicability of any measure such as this, based on single values, depends on random distribution of the objects. The measure for this condition is

$$t = \frac{\sigma - \sqrt{M}}{\sqrt{\frac{M}{2n}}}$$

where " σ " and " M " are the standard deviation and mean, respectively, of counts in a given number, " n ," of similar areas. If " t " is less than two the distribution satisfies the condition of randomness.

THE OCCURRENCE OF EXOERYTHROCYTIC SCHIZOGONY IN VARIOUS AVIAN MALARIAS DURING BLOOD PASSAGE

Exoerythrocytic schizonts have been sought in dry smears of tissues from birds infected with 4 strains of *P. relictum*. No exoerythrocytic stages were found in 12 birds (9 passages) infected with the Whitmore strain (1*W*), 4 (4 passages) with the Virginian strain (1*V*), and 3 each (3 passages) with the two undesignated strains. Similarly, exoerythrocytic stages were not found in 4 birds (3 passages) infected with the bluebird (9*J*) strain of *P. hexamerium*, 9 birds (6 passages) with the Sergeant (4*A*) strain of *P. rouxi*, and 3 birds (3 passages) with the Kikuth strain (6*G*) of *P. circumflexum*.

In 8 strains of *P. cathemerium* no exoerythrocytic schizonts were found during blood passage. These strains are the

Hartman (3*H*) strain (17 birds of 11 passages), the single cell (3*H*1)* strain (4 birds of 4 passages), strain 3*H*1-1* (4 birds of 4 passages), strain 3*H*1-2* (6 birds of 5 passages), the Hackett (3*D*) strain (5 birds of 4 passages), the gametocyteless 3*D*1 strain (6 birds of 5 passages), and strains 3*M*1* (6 birds of 6 passages) and 3*M*2* (10 birds of 8 passages). One bird each of the first canary passage of the two undesignated strains showed exoerythrocytic stages, and birds infected with strains 3*A* (grackle), 3*M* (magpie) and 3*T* (Wolfson) regularly exhibited exoerythrocytic schizogony. These last 5 strains are highly virulent for canaries, while the first 8 strains are considerably less pathogenic.

In the robin (1*R*) strain of *P. relictum* var. *matutinum*, exoerythrocytic schizonts were readily detected. In strain 5*E* (Taber sparrow) of *P. elongatum* the stages described by Huff and Bloom¹⁴ were abundant, but the large exoerythrocytic schizonts seen in other species were not found.

Six strains of *P. cathemerium* were examined for exoerythrocytic schizogony in birds inoculated with sporozoites. All of the strains thus investigated showed exoerythrocytic stages. These include not only strains 3*M* and 3*T* and the two undesignated strains, all of which showed exoerythrocytic schizogony during blood passage, but also strains 3*H* and 3*D*, which were negative during blood passage.

TISSUE DISTRIBUTION OF EXOERYTHROCYTIC SCHIZONTS THROUGHOUT THE COURSE OF SPOROZOITE-INDUCED INFECTIONS WITH *P. CATHEMERIUM*

The observations reported here are based principally on tissue sections of 7

* See materials and methods for descriptions of these strains.

birds inoculated with sporozoites of strain 3T and killed 2, 4, 7, 9, and 10, or dying 13 and 14, days after inoculation, 6 birds inoculated with sporozoites of strain 3H and killed 1, 2, 4, 7, 10 and 11 days after inoculation; 1 bird dying 11 days after inoculation with sporozoites of strain 3M; and 1 bird dying after the 8th day of infection with sporozoites of one undesignated strain. Supporting data are presented from dry smears of the tissues of 14 birds killed at various intervals after inoculation with sporozoites of strain 3T. For comparison observations are reported from tissue sections of 1 bird dying on the 15th day after inoculation with blood containing parasites of strain 1R of *P. relictum* var. *matutinum*.

Sections of the following tissues were studied: cerebrum and cerebellum, ventricles of the heart, pectoral muscle, skin, proventriculus, gizzard, duodenum, small intestine, colon, pancreas, adrenal, kidney, ovary, liver, abdominal fat, bone marrow and lung.

In the birds killed early in the infection exoerythrocytic stages occurred predominantly in macrophages of the liver (Kupffer cells), spleen (littoral cells of the sinuses, free macrophages and reticular cells of the red pulp, and, more rarely, reticular cells of the white pulp) and bone marrow (littoral cells of the sinuses, free macrophages and reticular cells of the myeloid tissue). Occasional parasites occurred also in the littoral cells lining the sinusoids of the adrenal. About half the exoerythrocytic schizonts in these organs were not determinably in cells. Whether they were actually extracellular, however, was not clear from the preparations. At least, no indisputably extracellular stages have been observed.

During the acute peripheral blood infection local accumulations of free macrophages and smaller polyblasts oc-

curred in larger vessels throughout the body of the bird. These were shown in the lung by Taliaferro and Cannon²⁹ and have been mentioned by Schulemann.²⁵ The cells may be in vascular emboli or apparently free in the vessels. Exoerythrocytic schizonts were seen in these macrophages wherever they occurred. Polyblasts resembling monocytes were rarely infected. Parasites not demonstrably intracellular were notably scarce in these locations, and here again no certainly extracellular schizonts have been seen.

Most of the cells listed above are actively phagocytic. They are, in fact, the cells involved in destruction of erythrocytic stages of malaria (see Cannon and Taliaferro¹ and Taliaferro and Mulligan³⁰). However, there was no clearcut indication of digestion of the exoerythrocytic schizonts despite the fact that many of the infected macrophages contained malarial pigment and the debris of erythrocytes and erythrocytic parasites (pl. 1, figs. 1 and 5). Some of the cells mentioned, it should be noted, show little or no activity in the defense against malaria. The littoral cells of the spleen rarely ingest infected erythrocytes. The reticular cells of the white pulp of the spleen do not ordinarily take up infected erythrocytes, but this is probably because they seldom come in contact with the circulating blood. A large group of functional macrophages have not been found infected. They are the pericytes lining capillaries throughout the body and the histocytes of the connective tissues. These cells do not normally come into direct contact with the circulating blood, and it is unlikely that they are exposed to infection. It should be noted that they phagocytose malarial parasites in the exceptional circumstances when infected erythrocytes come in contact with them (Taliaferro and Mulligan³⁰).

A marked change in the localization of exoerythrocytic schizonts occurred during the course of infection. In the series of birds infected with strain *3H*, exoerythrocytic schizonts, readily detectable in birds killed earlier, were not found in those killed on the 10th and 11th days of infection. In the other strains exoerythrocytic stages continued to appear in abundance but their distribution was strikingly different from that seen early in the infections. The new localization was found in the single birds killed late in infections with strain *3M* and one undesignated strain as well as in all birds of the first series of strain *3T* (studied in sections) after the 10th day and all those of the second series (studied in dry smears only) after the 7th day. Parasites continued to develop in large numbers in macrophages and organs rich in macrophages. In addition, however, they rapidly became abundant in endothelial cells and the lumens of capillaries throughout the body. In some birds killed early in infections with both strain *3T* and strain *3H* rare parasites had been detectable in capillaries and endothelial cells. They were, however, insignificant in number compared to those occurring in macrophages. In the later stages of infection capillaries and endothelium were equal in importance with the macrophages as a site for the development of exoerythrocytic schizonts.

About 87% of the parasites in these new locations were not determinably intracellular. The fact that there was no evidence of stoppage of blood flow would suggest that the parasites were attached to the wall of the capillary, whether or not they were actually contained in cells. In no case have parasites been observed which were determinably extracellular. Usually they appeared to fill the lumen of the capillary so that, if they were intracellular, the outlines of

the infected cell would not be clear. Occasionally they showed no contact with the sides of the capillary, but the possibility cannot be excluded that they bulged into the lumen from above or below. By indicating that these parasites might be intracellular, the writer has no intention of giving the impression that they probably were intracellular. Any of 3 possibilities is equally compatible with the appearance of the sections: they are actually extracellular; they are contained in the endothelial cells lining the capillaries; or they are contained in the cytoplasm of some other types of cells, the nuclei of which have been destroyed or expelled. The type of appearance presented is illustrated by plate 1, figure 4, showing a schizont and a segmenter which cannot be assigned to definite cells.

About 13% of the exoerythrocytic schizonts in these locations, however, were clearly contained in the cytoplasm of ordinary endothelial cells. This was most easily seen in larger vessels, the sides of which did not confuse the picture, but even in capillaries it was often possible to say with assurance that some of the parasites were in endothelial cells. This situation is illustrated in plate 1, figures 2, 3, and 7. Despite previous reports (discussed below), exoerythrocytic schizonts occurred in endothelium and capillaries not only in the brain but also, and often abundantly, in every tissue which has been studied. These tissues (listed above) include various glands, nervous tissue, 3 types of muscle, lung, and several types of connective tissue. There was no reason to doubt that all tissues of the body were infected in this way.

Ordinary endothelium is not normally phagocytic, although numerous claims have been made that erythrocytic malarial parasites are ingested by endothelial cells (see Taliaferro²⁸ for

discussion of these claims). Except rarely in sinusoid-like capillaries of the kidney, there was no indication from the tissues studied here that the endothelial cells infected with exoerythrocytic schizonts were phagocytic for erythrocytic parasites.

The observations on 1 bird dying late in the infection with *P. relictum* var. *matutinum*, admittedly inadequate as a study of this variety, are presented here because they showed only qualitative differences from the observations on *P. cathemerium*. One pseudo-eosinophil myelocyte of the spleen was found which was infected with a non-pigmented schizont (pl. 1, fig. 8). A great majority of the parasites were in capillaries and ordinary endothelium. Macrophages were infected only exceptionally, and, as would be expected from this fact, few parasites were to be found in the spleen, liver and bone marrow. In tissue smears, the parasites appeared to be abundant only in the brain and, to a less extent, the kidney. In sections, however, parasites were abundant throughout the body except in the organs with little capillary circulation, the liver, spleen and bone marrow. As in the infections with *P. cathemerium*, no tissue examined was unparasitized.

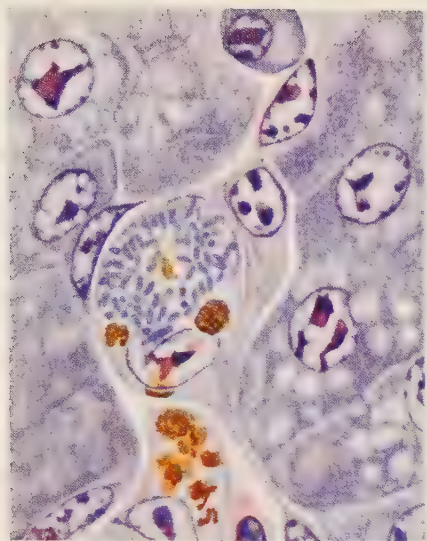
In order to clarify the relationship between the intensity of exoerythrocytic schizogony in various cells and tissues

and the course of infection in general, a quantitative estimation of the abundance of parasites in tissue sections has been attempted. Figure 1 presents the data obtained. The 7 canaries indicated were inoculated intravenously, each with a whole blood suspension representing about $8\frac{1}{2}$ pairs of salivary glands of infected *Culex pipiens*. These mosquitoes had been fed 17 to 22 days previously on blood containing numerous gametocytes of strain 3T of *P. cathemerium*. The upper curves represent counts of erythrocytic parasites in peripheral blood smears, expressed as the number of parasites per oil immersion field (Spencer 1.8 mm. objective, 10X oculars). The probable error of these counts is 15% or less when the count is greater than 1 per field. The number of parasites per microscopic field is less accurate than counts on a given number of erythrocytes. However, it is adequate for the present purpose, which is merely to indicate the stage of infection. A circle around the figure for a particular day shows that the bird was killed for examination on that day. A square similarly indicates that the bird died spontaneously. The numbers of the birds so designated appear below the curve. The lower curves represent the number of exoerythrocytic stages per square millimeter of section (8 microns thick) in representa-

PLATE 1

All figures except no. 8 represent exoerythrocytic schizonts of *Plasmodium cathemerium* (strain 3T) in tissues of canaries infected by means of sporozoites. Fixation, Zenker-formol; stain, hematoxylin-eosin-azure II, celloidin sections. Magnification 2,500X.

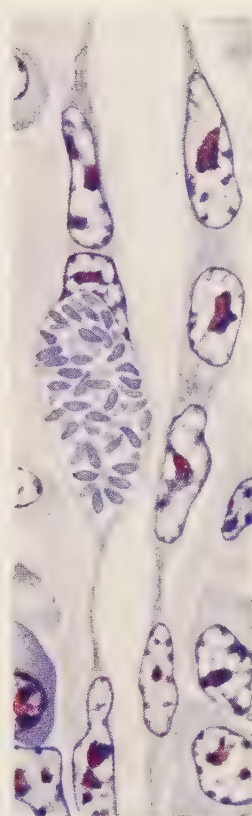
1. Portion of a liver sinusoid, showing phagocytosed pigment and a segmenting exoerythrocytic parasite in a Kupffer cell. 2. Capillary in the brain, showing an endothelial cell infected with a uninucleate parasite and a schizont. 3. Terminal arteriole in the spleen, showing a segmenting exoerythrocytic stage in an (ordinary) endothelial cell. 4. A schizont and a segmenting parasite, apparently extracellular, in the lumen of a capillary in heart muscle. 5. Free macrophage, from a vein in the kidney, containing a large exoerythrocytic schizont, a vacuole with pigment and a partially digested erythrocyte. 6. Portion of a liver sinusoid showing parasitized erythrocytes, a Kupffer cell containing pigment and a uninucleate exoerythrocytic parasite, and a lining cell infected with a schizont. 7. Two exoerythrocytic schizonts in an endothelial cell of a capillary in pectoral muscle. 8. Schizont in a pseudo-eosinophil myelocyte in the spleen of a bird infected with *P. relictum* var. *matutinum* (strain 1R).



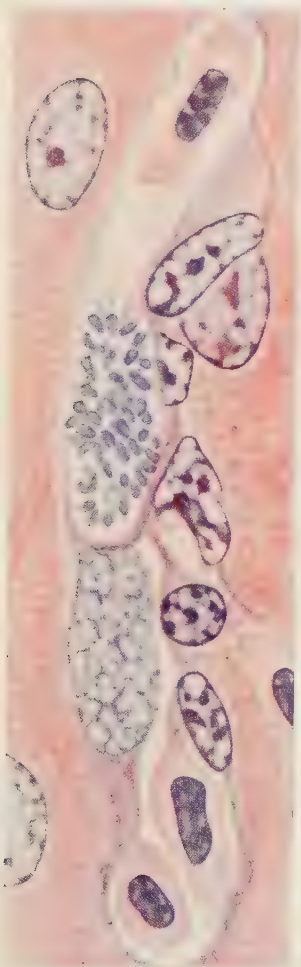
1



2



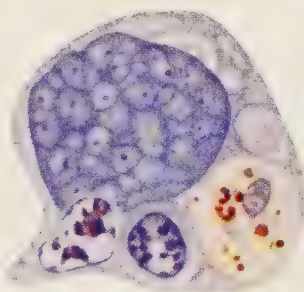
3



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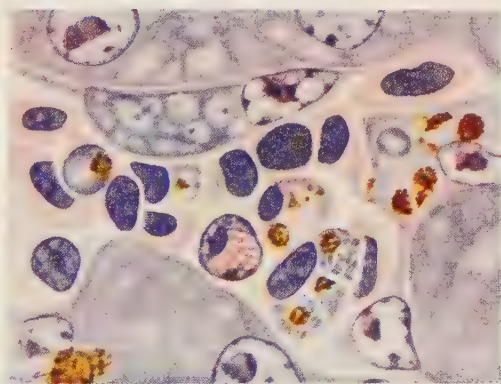


8

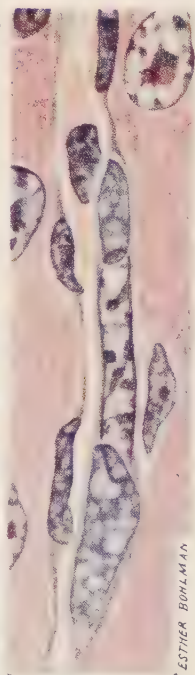


5

6



7



tive tissues. It should be particularly noted that the counts of parasites from tissue sections for any one day are based on 1 bird only, the bird indicated as

intensity of infection in tissues is novel, its reliability must be severely questioned. Certain obvious precautions have been taken. First, only tissues

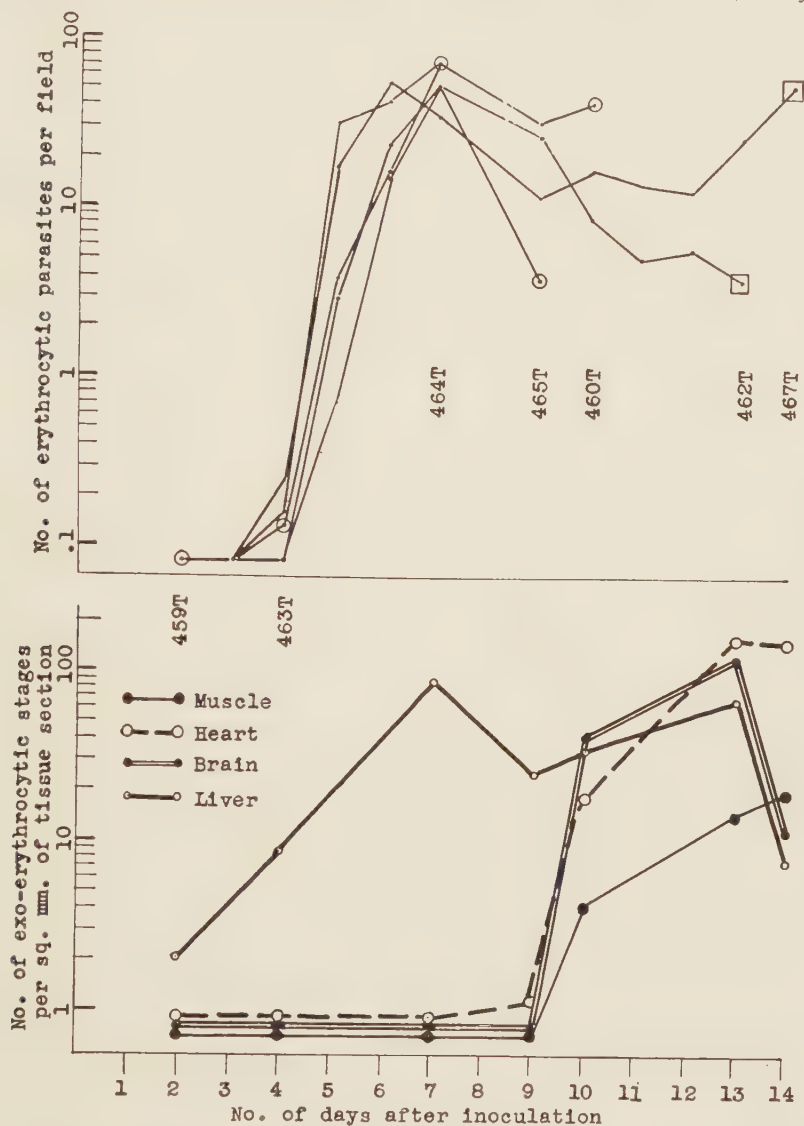


Fig. 1.—Distribution of exoerythrocytic parasites in tissue sections (below) compared with the course of peripheral blood infection (above). Circles or squares in the upper curve indicate that the bird was killed or died, respectively, on that particular day. Data from the tissues of these birds appear in the lower figure on the day of death of the bird.

killed on that day. Thus, each point on any of the curves represents a different bird.

Since this method of representing the

whose structure and cellular distribution are apparently homogeneous can be used. Second, those areas must be avoided in which occur large intravascu-

lar accumulations of macrophages, for these areas show a local preponderance of exoerythrocytic stages not representative of the tissue as a whole. Third, it must be demonstrated that the distribution of parasites in the tissue, excluding those in the intravascular macrophages just mentioned, is essentially random. In the enumeration of exoerythrocytic stages in most of the sections, the areas examined have been divided into a number of smaller areas of uniform size and the distribution of parasites among these smaller areas determined. In all cases the distributions fulfilled the condition that " t " be less than 2 in the formula given in "Materials and Methods." Similarly, in a smaller number of cases, the distribution of parasites among several widely separated areas of the tissue fulfilled the same condition. Finally, subjective impressions of the general intensity of infection in different areas of the same tissues were in reasonably good agreement, indicating that, always excepting the macrophage accumulations discussed above, there were not gross differences in abundance of the parasites in different regions.

Where the number of parasites is given as less than 1 per square millimeter, actual counts have been impossible; it can only be said that the parasites were rare or absent. Where the counts are between 1 and 5 the probable error, obtained by the technic outlined in "Materials and Methods," is less than 25%. Between 5 and 15 it is less than 20%; between 15 and 25 it is less than 15%; and above 25 it is less than 10%. It should appear from the above that the intensities of infection in the tissues studied are reasonably well represented by the recorded figures.

It may be seen from the graph that, while the exoerythrocytic stages in the liver increased in rough parallelism with

the peripheral blood stages, those in organs with a capillary circulation—brain, heart muscle and skeletal muscle—did not become abundant until late in the infection. Subjective estimations from other tissues, not amenable to quantitative treatment, indicated that in the spleen and bone marrow the exoerythrocytic stages, although less abundant than in the liver, showed an early increase in numbers, whereas in other tissues with a capillary circulation—alimentary tract, loose connective tissue, smooth muscle, pancreas and lung—the parasites did not become readily detectable until late in the infection, when they had become abundant in the brain and muscles. A change in the localization of exoerythrocytic stages in the liver also occurred. While they were found exclusively in littoral cells early in the infection, later some were seen in the endothelial cells of the central veins and the smaller vessels of Glisson's capsule. Similarly, all the exoerythrocytic stages seen in the spleen and bone marrow early in the infections were in macrophages or so located that they could not conceivably be in endothelial cells. Later, occasional parasites in these organs could be seen in endothelial cells, their rarity probably being attributable to the small amount of ordinary endothelium in the tissues.

Table 1 presents supplementary data from another series of birds inoculated with sporozoites of strain 3T. The intensity of infection with exoerythrocytic schizonts was estimated from dry smears of the tissues. The estimates are expressed in numbers of plus signs from 1 (barely detectable infection) to 4 (heavy parasitization). The data here are less reliable from a quantitative point of view, but they offer valuable support to the results discussed above. In the liver, spleen and bone marrow exoerythrocytic stages appeared on the

second day of infection. In the brain, on the other hand, parasites were not detected before the 6th day. Here the interval between the appearance of parasites in the liver and their appearance in the brain was shorter than in the series discussed above, but there was, nevertheless, a marked difference.

TABLE 1.—*Distribution of Exoerythrocytic Schizonts in Tissue Smears of Birds Inoculated with Sporozoites of P. cathemerium (strain 3T)*

Days after Inoculation	Bird No.	Bone Marrow	Spleen	Liver	Brain
2	414T	+	+	+	0
3	433T	0	+	+	0
	411T	+	+	+	0
4	417T	0	+	++	0
	429T	+	++	++	0
5	407T	++	++	++	0
	409T	++	++	+++	0
6	408T	+	++	+++	+
	412T	+++	+++	+++	+
	415T	+++	+++	+++	+
7	416T	+++	+	++	+++
	432T	+++	+	+++	+
	430T	++	++	+++	+++
	431T	++	+++	+++	+++

While the courses of infection within the above groups of organs were similar, differences in intensity of infection occurred. Thus, in the brain and heart muscle exoerythrocytic stages were usually more abundant than in the breast muscle (fig. 1) or other tissues with capillary circulation. In this case, the intensity of infection seems roughly correlated with the abundance of capillaries, but other factors may be involved. In a similar fashion the liver was usually more heavily parasitized than the spleen or bone marrow.

DISCUSSION

It should be emphasized that failure to find exoerythrocytic stages in examinations such as those described in the first section of this paper cannot exclude their occurrence in the strains studied. An example of this is that fact that

Manwell and Goldstein¹⁹ found exoerythrocytic schizonts in the Kikuth strain of *P. circumflexum*, whereas the present inadequate study and that of Kikuth and Mudrow¹⁷ failed to reveal them in the same strain. Negative results with strains 3H and 3D are significant, however, in view of the ease with which exoerythrocytic stages were found in them after mosquito passage. In the Hartman strain (3H) Hegner and Wolfson¹⁰ found no exoerythrocytic stages during blood passage, and Kikuth and Mudrow¹⁷ found them in small numbers only after mosquito transfer. Similar results were obtained by Kikuth and Mudrow¹⁷ with other strains of *P. cathemerium* and *P. relictum* and by Raffaele²² with *P. relictum*. In these two species, at least in a number of strains, it appears that exoerythrocytic schizonts occur after mosquito passage whether or not they can be found during blood passage. This phenomenon has been generally taken to indicate that the exoerythrocytic stages represent the first developmental phase of inoculated sporozoites. It must be emphasized, however, that mosquito passage might affect a strain of malaria in many ways (as, for instance, in the increased numbers of gametocytes shown by Huff¹⁸) and such changes, in strains adversely affected by long-continued blood passage, may be associated with the appearance of exoerythrocytic schizogony.

In this connection it is interesting that strains 3M1 and 3M2 of *P. cathemerium* have not shown exoerythrocytic schizonts, although these stages occur abundantly in the parent strain (3M). These strains, obtained in experiments aimed at the isolation of clons, arose from very small numbers of parasites. It is conceivable that strain 3M and the populations of parasites in birds inoculated with sporozoites are complexes of races. Rigorous selection, as by contin-

ued blood passage or by a single transfer of very few parasites, might eliminate those capable of developing exoerythrocytically. It should be noted that Coulston and Manwell⁷ found exoerythrocytic schizogony in a clon and several strains derived from few parasites of *P. circumflexum*. There is a noteworthy similarity between strains *3M*, *3A* and *3T* of *P.*

The observation of exoerythrocytic schizogony in strain *1R* of *P. relictum* var. *matutinum* agrees with previous reports. They were seen in another strain by Manwell¹⁸ and, after passage through ducks, in the woodthrush strain (*1T*) by Dobler.⁹ It seems likely that exoerythrocytic schizogony is a common phenomenon in this form.

TABLE 2.—Cells Reported Infected by Exoerythrocytic Schizonts

Cells ^a	Species			
	<i>P. relictum</i>	<i>P. circumflexum</i>	<i>P. gallinaceum</i>	<i>P. cathemerium</i>
Endothelial cells in the brain	(Rodhain) ^{23,24}	(Manwell and Goldstein) ¹⁹	(James and Tate, ^{15,16} DeCourt and Schneider) ⁸	Most parasites (Kikuth and Mudrow) ¹⁷
Endothelium elsewhere			In spleen and liver (James and Tate) ^{15,16}	
Reticulo-endothelial cells ^b	(Raffaele, ²¹ Taddia and Viero) ²⁷		In brain capillaries, etc. (James and Tate), ^{15,16} exceptionally (DeCourt and Schneider) ⁸	
Macrophages ^b	Kupffer cells (Rodhain) ²³	Fixed and free macrophages (Coulston) ⁶	Fixed macrophages (Corradetti), ⁴ many parasites in free macrophages (James and Tate), ¹⁶ histiocytes (Schulemann, ²⁵ Schulemann and Spies) ²⁶	Most intracellular parasites in the liver (Kikuth and Mudrow), ¹⁷ probably in tissue culture (Hegner and Wolfson) ¹¹
Septal cells in the lung			(James and Tate) ¹⁶	
Monocytes ^c	Often with phagocytosed pigment (Rodhain) ²³	Most parasites (Manwell and Goldstein) ¹⁹	(James and Tate, ¹⁵ Corradetti) ⁴	Most intracellular parasites in the lung (Kikuth and Mudrow) ¹⁷
Lymphocytes		(Manwell and Goldstein) ¹⁹		
Circulating leucocytes		Heterophils ^d (Coulston), ⁶ granulocytes (Manwell and Goldstein) ¹⁹	(James and Tate) ^{15,16}	A few uninucleate parasites in pseudo-eosinophils (Kikuth and Mudrow) ¹⁷
Extracellular in smears	Most parasites (Raffaele, ^{21,22} Rodhain, ²³ Corradetti) ⁴		Most parasites (Corradetti) ⁵	Most parasites except in the brain (Kikuth and Mudrow) ¹⁷

^a The names of cell types given are those used by the authors cited.

^b Reticulo-endothelial cells as used by some authors include ordinary endothelial cells and hence may not be equivalent to macrophages.

^c Macrophages are probably meant in many cases.

^d By "heterophils" are probably meant pseudo-eosinophils.

^e As pointed out in the text, James and Tate undoubtedly mean littoral cells when they speak of "endothelial cells" in the spleen and liver.

cathemerium. All regularly show exoerythrocytic schizogony during blood passage and all are markedly virulent for canaries. Wolfson³¹ reported that strain *3T* exhibited maximum segmentation in the peripheral blood earlier in the afternoon than did other strains of *P. cathemerium*. This was observed also in strain *3A* by Redmond (unpublished) and in strain *3M* by Coatney (unpublished) and by the writer.

In regard to the types of cells infected, if a certain latitude in terminology be admitted, there is no serious disagreement in the literature with the present finding that many of the exoerythrocytic stages are in macrophages. Reference to table 2 shows that macrophages and reticulo-endothelial cells have been widely reported as infected. James and Tate^{15,16} have reported infection of "endothelial cells" in the spleen and

liver. While it is true that in the infections discussed above the small amount of ordinary endothelium in these organs may be infected, it is likely that the authors cited refer to littoral cells or *special* endothelium. The "monocytes" of many investigators are undoubtedly free macrophages. The histiocytes of Schulemann²⁵ are macrophages of various organs. Of the histiocytes of Schulemann and Spies,²⁶ however, some are probably true histiocytes of the subcutaneous connective tissue and most undoubtedly are macrophages accumulated locally following the insertion of sterile sponges. These authors injected sporozoites directly into such accumulations of cells and found abundant exoerythrocytic stages in them 24 to 65 hours later. It will be recalled that tissue histiocytes were not found infected in the present study. The fact that they become infected when the parasites are inoculated directly into the loose connective tissue supports the view that lack of opportunity prevents their infection normally.

Chortis^{2,3} suggested that exoerythrocytic schizonts were able to develop in cells which actively destroy erythrocytic parasites, because of lowered "phagocytic function" of the macrophages. Coulston⁶ reported that in *P. circumflexum* infections, few exoerythrocytic stages were found when much malarial pigment was present in the macrophages. Rodhain,²³ on the other hand, stated definitely that infected macrophages might contain large quantities of phagocytosed pigment, and Schulemann²⁵ and Schulemann and Spies²⁶ found injected dye or colloidal metals in the parasitized cells. Clearly then, at least in some cases, the infected macrophages are actively phagocytic. It has been pointed out above that a similar situation occurs in the infections studied here. Plate 1, figures 1, 5, and 6 show infected macro-

phages containing malarial pigment. This is not proof that the cells involved retain their ability to digest phagocytosed erythrocytic parasites, since free pigment is apparently often taken up by these cells. However, there is evidence of active digestion in some of the infected cells. Plate 1, figure 5 illustrates this, and such pictures of partially digested erythrocytes and erythrocytic parasites are common. There is at present no satisfactory explanation for the fact that exoerythrocytic schizonts can develop in cells which are at the same time capable of digesting erythrocytic parasites. However, it should be pointed out that in any case the hypothesis of lowered phagocytic power cannot constitute a complete explanation of exoerythrocytic schizogony, since the parasites occur not only in macrophages but also in ordinary endothelium, which is not normally phagocytic at any time.

While there is little dispute on the location of most parasites in the spleen, liver and bone marrow, the present findings disagree with previous reports on the cells infected in other organs. It has been pointed out above that James and Tate¹⁶ undoubtedly mean littoral cells when they report infection of "endothelial cells" in the spleen and liver. All other investigators indicate that except in the brain the infected cells are macrophages. In the present series, it has been shown that in all organs studied except the spleen, liver, bone marrow and adrenal the exoerythrocytic parasites occur in two distinct locations, intravascular accumulations of macrophages on the one hand, and capillaries and vascular endothelium on the other hand. Capillaries are well shown in smears of the brain but they are poorly preserved in smears of other tissues with a capillary circulation. Such smears, of muscle or lung, for instance, consist principally of blood cells contained in the tissue. It

is likely that the only intracellular exoerythrocytic stages seen in such preparations are those occurring in intravascular macrophages. Thus, not only would smears (except of the brain) fail to reveal the numerous parasites reported here in capillaries and endothelial cells of all organs, but it would also appear that the distribution of exoerythrocytic schizonts was capriciously variable in organs with a capillary circulation. The frequent reports of irregular abundance of exoerythrocytic stages in some organs are probably traceable, as is the general failure to observe parasites in capillaries and endothelium throughout the body, to the study of tissue smears rather than of sections. In the strains studied here, at least, the parasites in intravascular macrophages are only locally abundant and constitute a minor part of the picture in organs with a capillary circulation.

The discrepancies between Schlemm's report and the present results are more difficult to reconcile. He states, on the basis of unpublished work by Knoche, that the exoerythrocytic stages in organs with a capillary circulation are either in free macrophages (histiocytes), as described above, or extracellular in the lumens of capillaries. It has been pointed out that in the infections studied here it is often difficult to demonstrate parasites which are unquestionably in ordinary endothelium. However, it cannot be doubted that some, at least, of the parasites are in endothelial cells. Rodhain^{23,24} has indicated that in *P. relictum* of penguins exoerythrocytic schizonts occur in endothelial cells of capillaries and larger vessels. Some figures of *P. gallinaceum* published by James and Tate¹⁶ suggest parasites in the endothelium of brain capillaries. This would indicate that the difference between the present findings

and those of Knoche are not traceable to differences in the species studied.

The distribution of exoerythrocytic schizonts in *P. relictum* var. *matutinum* differs only in degree from that in *P. cathemerium*. Manwell¹⁸ has reported that the exoerythrocytic schizonts in a strain studied by him show a striking predilection for the brain. This would be the conclusion in the present study also if only smears had been examined, but it has been noted that from sections an entirely different conclusion is obtained. It was suggested above that in smears only the exoerythrocytic schizonts in intravascular macrophages are seen. In the one infection with *P. relictum* var. *matutinum* studied here so few schizonts were found in macrophages that smears of capillary tissues might show no parasites at all, even when the endothelium and capillaries were abundantly infected.

In addition to the one pseudo-eosinophil myelocyte found infected in this study rare schizonts were found by Kikuth and Mudrow¹⁷ in pseudo-eosinophils and by Coulston⁶ in heterophils (pseudo-eosinophils). It should be borne in mind that occasional phagocytosis of malarial parasites by heterophils has been reported in various malarias.³⁰ Various cell types reported infected by others have not been found parasitized here. As has been stated, very rare parasites occur in small polyblasts, and some of these resemble monocytes. Parasites have not been found in small or medium lymphocytes. One schizont was seen in a large (tissue) lymphocyte, but, as Huff and Bloom¹⁴ have noted, functional macrophages are occasionally indistinguishable morphologically from hemocytoblasts. Lymphocytes, reported parasitized by Manwell and Goldstein,¹⁹ have not been found infected in the present study.

Figure 2 represents schematically a

comparison of the abundance of infection in various cells parasitized by *P. elongatum* or the strains of *P. cathe-*

ing abundance of parasites. The data on *P. elongatum* are based on the percentages, given by Huff and Bloom,¹⁴ of

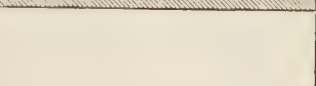
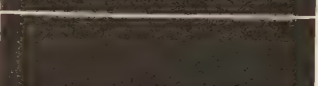
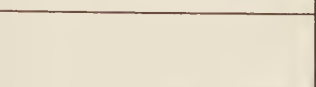
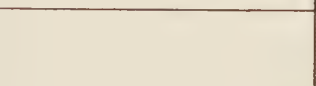
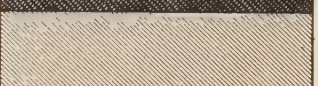
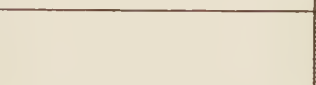
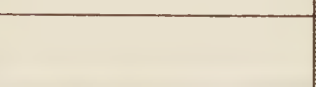
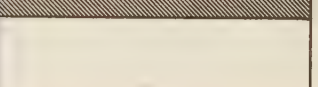
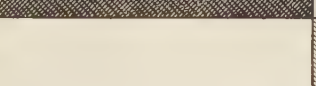
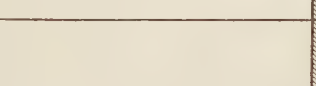
Cells	<u>Plasmodium</u> <u>cathemerium</u>	<u>Plasmodium</u> <u>elongatum</u>
Erythrocytes		
Normoblasts		
Polychromatophil erythroblasts		
Basophil erythroblasts		
Hemocytoblasts		
Lymphocytes		
Monocytes		
Plasma cells		
Macrophages		
Ordinary endothelium		
Granulocytes		
Thrombocytes		

Fig. 2.—Types of cells infected by *P. cathemerium* and *P. elongatum*. The relative abundance of parasites in a particular cell type is indicated by the density of shading.

merium studied here. Absence of shading indicates that the type of cell is rarely or not at all infected, and increasing degrees of density indicate increas-

parasites in various cells in wet-fixed smears of bone marrow, spleen and liver. The writer has specifically sought evidence of endothelial infection in dry

smears of the brain and, like Huff and Bloom,¹⁴ has found no such indication. The data on *P. cathemerium* are based on the present study of sections of various tissue. In neither case are the data strictly quantitative, since the susceptible cells vary in number during the infections and since, in infections with *P. cathemerium*, the relative intensity of parasitism of various cells differs markedly at different times. The scheme is intended only to show, in a general way, the susceptibility of various cell types to the two species of parasites.

Certain gross differences between the two species may be pointed out. In infections with *P. elongatum* the predominantly infected cells are erythroblasts and, to a less extent, hemocytoblasts (large tissue lymphocytes). Parasites are not known to occur in ordinary endothelium. In *P. cathemerium*, on the other hand, the erythrocytes are most commonly infected, with macrophages and endothelial cells next in importance. Other, less striking, differences are evident from the chart. It seems clear that the two species are considerably different in the types of cells they most commonly infect. For the purpose of clarity it is suggested that they be designated respectively *elongatum*-type and *gallinaceum*-type exoerythrocytic stages.

A comparison of plate 1 with Huff and Bloom's plate 1 of *P. elongatum* reveals two interesting facts. First, while *P. elongatum* characteristically shows a distinct vacuole around the parasite in all stages except those in granulocytes and erythrocytes, only the segmenters of *P. cathemerium* show such a vacuole at all distinctly. Second, the general appearance of schizonts in both species is strikingly similar in preparations fixed with Zenker-formol and stained with hematoxylin-eosin-azure II. Huff and Bloom¹⁴ pointed out the regular occurrence of reddish granules surrounded by

large vacuoles in the uninucleate stages and younger schizonts of *P. elongatum*. They considered that the central granule was the nucleus. The figures presented here show, both in an erythrocytic stage (fig. 6) and in exoerythrocytic stages, very similar appearances. Furthermore, the merozoites of *P. cathemerium* are somewhat elongate though not to the extent seen in *P. elongatum*. It is noteworthy that with this technic of preparation there is this similarity in morphology between erythrocytic stages and both *elongatum*-type and *gallinaceum*-type exoerythrocytic stages.

The locations in which exoerythrocytic schizogony occurs are clearly differentiated into two basic types, those in which the intracellular parasites are in macrophages (spleen, liver, bone marrow and intravascular accumulations of macrophages), and those in which the parasites are in vascular endothelium or the lumens of capillaries (blood vessels throughout the body lined with ordinary endothelium). The general similarity in courses of infection observed in the spleen, liver and bone marrow on the one hand and the organs with a capillary circulation on the other hand indicates that physiological differences in the type of circulation and in the nature of the cells lining the blood vessels are important in determining the location of the exoerythrocytic parasites. It appears that undue emphasis has been put on the susceptibility of specific organs, whereas it seems more likely that the type of cells in contact with the circulating blood is the major factor. It is difficult to suggest any explanation involving the physiology of particular organs for the fact that the course of infection in liver, spleen and accumulations of macrophages in the larger vessels of the lung and kidney is markedly different from the course of infection in the capillaries of the pancreas, brain,

heart muscle and lung. A simple interpretation is possible, however, if it is recognized that the cells infected in the first group of tissues (macrophages) are physiologically distinct from those parasitized in the second group (ordinary endothelial cells). This should not obscure the fact that there are significant differences in the intensity of infection in different tissues within these groups. Thus, it has been pointed out that exoerythrocytic schizonts are found more abundantly in the brain and heart muscle than in, for instance, pancreas and skeletal muscle. In this case the difference may be traceable to the greater abundance of capillaries in the brain and heart. In the case of the spleen, liver and bone marrow, however, the differences are not so easily explained. The present study shows, as Kikuth and Mudrow¹⁷ and others have previously reported, that the liver is usually more heavily infected than the spleen or bone marrow. The bone marrow contains, per unit volume, fewer functional macrophages than the liver, but the spleen contains many more. Clearly, some differences other than that in abundance of macrophages are involved, but it cannot be said whether the important factors are the type of blood in the liver (principally venous), unknown differences in the nature of the macrophages, or many other possible dissimilarities between these organs.

Kikuth and Mudrow¹⁷ found that after inoculation with sporozoites of *P. cathemerium* exoerythrocytic schizonts appeared earlier in the liver, spleen and bone marrow than in the brain. The results of the present study justify the assumption that observations on brain smears are a fair indication of the situation in capillaries and endothelium throughout the body. Thus, the present results appear to agree with the findings of Kikuth and Mudrow. In *P. galli-*

naceum both James and Tate¹⁶ and Kikuth and Mudrow¹⁷ found exoerythrocytic stages in the brain before they were detectable in the liver, whether the infections were produced by sporozoites or infected blood. This apparent difference between *P. cathemerium* and *P. gallinaceum* may be traceable to specific differences, but it should be pointed out that in the experiments with *P. gallinaceum* the incubation periods were long (7 to 15 days) and changes may have occurred in the birds before parasites were observed.

Raffaele²¹ and Kikuth and Mudrow,¹⁷ with strains of *P. cathemerium* and *P. relictum* which showed no exoerythrocytic schizonts during blood passage, found that after mosquito transfer these stages appeared in small numbers early in the infections. In such strains exoerythrocytic stages are rare or absent in brain smears (Raffaele,²¹ Corradetti⁵) and in capillaries and endothelium of all tissues (data presented here on strain 3H).

The present observations suggest a clarification of one of the most difficult questions concerning exoerythrocytic schizogony. Among the facts which have fostered the hypothesis that exoerythrocytic schizogony was related to the development of sporozoites are the very early appearance of exoerythrocytic stages after sporozoite inoculation and their occurrence after mosquito transfer in strains which do not show them during blood passage. The picture has been complicated by the fact that some strains continue to exhibit exoerythrocytic schizogony after many blood passages. However, it has been shown that the exoerythrocytic stages of *P. cathemerium* and *P. relictum* occurring very early after sporozoite inoculation develop almost exclusively in macrophages or, at least, in organs whose smaller blood vessels are lined with

macrophages. The exoerythrocytic schizonts appearing in some strains late after either blood or sporozoite inoculation show a wider distribution, occurring abundantly also in capillaries and endothelial cells of all organs. It is suggested that the early exoerythrocytic stages are related to sporozoite development whereas the later stages indicate a physiological change, produced in the host by certain strains of parasites, whereby erythrocytic merozoites are enabled to develop exoerythrocytically. Any of numerous effects of the disease might be responsible for this hypothetical change in susceptibility of the host. Other explanations are possible, but the hypothesis that there are at least two different phenomena involved in the occurrence of exoerythrocytic schizogony offers a simple interpretation of a number of confusing facts.

SUMMARY

Exoerythrocytic schizonts have been found in birds infected with 5 virulent strains of *P. cathemerium*, including strain 3T (Wolfson). They have been sought without success in 8 other strains, all less pathogenic than the strains which show exoerythrocytic stages. In 2 of these strains, however, the Hartman and Hackett strains, exoerythrocytic schizogony appeared after mosquito passage. The other 6 were not tested.

Strain 1R of *P. relictum* var. *matutum* has also shown exoerythrocytic schizogony. Four strains of *P. relictum* and 1 strain each of *P. hexamerium*, *P. rouxi*, and *P. circumflexum* have been examined for exoerythrocytic schizonts without success. In a strain of *P. elongatum* the stages described by Huff and Bloom were seen, but the large schizonts characteristic of other avian malarias have not been observed.

In tissue sections of birds inoculated

with sporozoites of various strains of *P. cathemerium* the exoerythrocytic schizonts occurred almost exclusively in macrophages of the liver, spleen and bone marrow during the earlier stages of infection. Late in the infections the parasites became abundant in ordinary endothelium and capillaries throughout the body. Numerous parasites also occurred locally in intravascular accumulations of macrophages in all organs. It is suggested that the early stages in macrophages are related to sporozoite development while the later stages in macrophages, endothelial cells and capillaries arise as a result of changes in the susceptibility of the host. Regardless of the explanation, early and late schizogony probably involve different processes.

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